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(54) Title: RIBOZYME-BASED COMPOSITIONS FOR THE MODIFICATION OF CUTANEOUS PHENOTYPES ASSOCIATED WITH AGING AND OTHER CONDITIONS OF THE SKIN AND HAIR

(57) Abstract

This invention relates to methods for the generation of nucleic acid compositions for the treatment of conditions affecting the skin and related structures. Nucleic acid molecules such as ribozymes which specifically interact with and modify the expression of cutaneous biological molecules can be developed as effective, safe, therapeutic or cosmetic reagents. These ribozymes can be used for the treatment of disorders that include: aging, environmental insult, inflammation, and genetic predisposition. The methods used to develop these reagents are described.

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RIBOZYME-BASED COMPOSITIONS FOR THE MODIFICATION OF CUTANEOUS PHENOTYPES ASSOCIATED WITH AGING AND OTHER CONDITIONS OF THE SKIN AND HAIR.

BACKGROUND OF THE INVENTION

Throughout this disclosure, scientific publications are referenced by short citations of the authors and the year of publication. The corresponding complete citations for these publications are provided in the References section preceding the claims.

Ribozyme-Based Therapeutics and Their Potential for the Treatment of Skin Ailments.

The rapidity with which the sequences of genes can be ascertained has allowed the design of complementary oligonucleotides (oligomers) that can interact through specific base pairing with a gene or its mRNA. This interaction can modify gene expression by interfering with the transcription or translation of the targeted DNA or RNA (antisense molecules), and in the case of ribozymes, by cleaving the targeted sequence (Murray, J.A.H. and Crockett, N., 1992; Symons, R.H., 1991).

Ribozyme technology combines the base pairing-specific targeting of the "antisense" approach with the ability of several different classes of RNA molecules to act like enzymes. Ribozymes are RNA molecules which naturally cleave, or can be readily engineered to cleave, other RNA molecules (Symons, R.H., 1991). Unlike antisense molecules which act stoichiometrically, ribozymes behave catalytically, like true enzymes, in that the ribozyme molecule is unchanged after cleaving its target RNA and can participate in additional cleavage reactions.

There are at least five classes of ribozymes: 1) Plant viroid and satellite RNAs containing the so -called "hammerhead" motif; 2) RNase P or its RNA component; 3. Tetrahymena group I intron-like

RNAs; 4) The "hairpin"-type motif of tobacco ringspot virus minus strand satellite RNA; and 5) The catalytic motifs of hepatitis delta virus. With the exception of RNase P, ribozymes are normally self-cleaving (*cis*-cleaving), and must be engineered to cleave other molecules (*trans*-cleaving). Such engineering involves the isolation of that subset of the entire self-cleaving molecule's sequence which contains the enzymatic activity (the catalytic core) from that part of the molecule which contains the sequence(s) that are cleaved.

The smallest *trans*-cleaving ribozymes are the "hammerhead" class (Forster, A.C. and Symons, R.H., 1987; Uhlenbeck, O.C., 1987; Haseloff, J. and Gerlach, H.L., 1988). These ribozymes, ranging from 25-50 nucleotides in length, consist of a catalytic core of about 11 nucleotides, with the remainder of the residues largely participating in the formation of 3 stem structures (see Figure 1). In this "hammerhead" structure, the ribozyme residues of stems I and III act as internal guide sequences (IGS), hybridizing to the complementary target (substrate) RNA sequence. The only sequence requirement of the substrate is that it contains the sequence NUX (where N=any nucleotide, and X=any nucleotide except G) between the flanking sequences targeted by base pairing. In the presence of a divalent cation (usually Mg^{++}), cleavage of the substrate occurs immediately after the NUX trinucleotide, generating fragments with 5' hydroxyl and 2',3' cyclic phosphate groups (reviewed by Symons, R.H., 1992).

Several recent studies have demonstrated that the introduction of *trans*-cleaving ribozymes (primarily of the hammerhead type) into cells, results in a reduction of targeted mRNA and/or affected protein levels (Saxena, S.K. and Ackerman, E.J., 1990; Steinecke, P. et al., 1992; Huillier, P.J.L. et al., 1992; Sioud, M. et al., 1992; Yu, M. et al., 1993). Numerous studies have demonstrated the low toxicity of nucleic acid reagents in cell culture (Vlassov, V.V. and Yakubov, G. 1991), in mice (Agrawal, S. et al., 1988; 1989a; 1989b; Sarin, P.S. et al., 1988; Stewart, R. et al., 1992; Zon, G., 1989), and in humans (Hutcherson, S.L. et al., 1993). These results indicate

that these molecules can potentially be developed as safe, specific, human therapeutic agents.

Although affecting an estimated 15% of the population, the treatment of skin disorders has not been addressed adequately by the medical or pharmaceutical research communities. Disorders of the skin and hair, while rarely life-threatening, have medical and cosmetic ramifications. Alterations of the skin and hair as a result of aging, UV-induced damage (photoaging and photocarcinogenesis), metabolic disorders (such as male pattern baldness, female hirsutism, and acne), and psoriasis, fall within a category for which treatment could be described as "cosmesis through medical therapy" (Weiss, J.S., et al., 1991).

While antisense approaches to viral cutaneous diseases are now in clinical trials, research involving nucleic acid-based pharmacological reagents has been primarily directed towards developing systemic therapeutics against pathologies such as leukemia, hepatitis, and AIDS. It is generally accepted that systemic delivery will require fairly large amounts of these therapeutics and raises concerns about cost and the possibility of eliciting an immune response. The skin is the most accessible organ of the body, and nucleic acid-based therapeutics developed for the treatment of skin ailments can be applied topically and locally. This is advantageous because: 1) it allows relatively easy access to the cells one wishes to target; 2) it is likely to demonstrate far fewer complications than systemic administration; and 3) it should prove more cost effective, requiring much lower amounts than systemic dosages.

Many of the pharmacological agents currently used for treatment of skin disorders are either inadequate or have such diverse effects that adverse reactions are common. In some cases the mechanisms of action of these reagents are not clearly understood. The development of ribozyme reagents, with their inherent specificity and negligible toxicity, could overcome these disadvantages. In addition, the use of these agents to reverse cutaneous conditions which are more cosmetic, rather than medical, such as aging-related changes and hair loss (see below),

represents a completely novel application of nucleic acid-based therapeutics.

SUMMARY OF THE INVENTION

This invention concerns the production of oligonucleotides and their modified derivatives designed to reduce the levels of specific biological molecules present in the skin. In a specific embodiment of the invention the oligomers are ribozymes. The oligomers of the invention are used in inventive methods of treatment to counteract a variety of medically and cosmetically undesirable cutaneous conditions, such as intrinsic and actinic aging, psoriasis, alopecia, hirsutism, acne, and pigmentation, which are responsible for alterations in the functioning and appearance of the skin and related structures, and are due to aging, environmental insult, inflammation, or genetic predisposition. The inventive methods disclosed here comprise contacting the skin of the patient with an effective amount of a composition comprising an oligomer and a suitable carrier such as liposome which inhibits the expression of a gene product associated with the cutaneous phenotype.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1

General Structure of "Hammerhead" Ribozyme

A schematic representation of a "hammerhead" ribozyme is shown. The sequence of the catalytic core of the ribozyme is indicated by bold letters while the consensus cleavage site in the target is denoted by the italicized "NUX". Stem 2 is formed by intramolecular base pairing within the ribozyme sequence. Stems 1 and 3, in contrast, are formed by the hybridization of guide sequences from the ribozyme, to complementary sequences found in the target substrate. The ribozyme is shown as a bold line and the target as a light line.

Figure 2

Interstitial Collagenase mRNA-specific "Hammerhead" Ribozyme

A "hammerhead" ribozyme (ICRZ1) and target substrate (interstitial collagenase RNA) complex is shown. The partial sequence of the substrate RNA is indicated by italics. Ribozyme guide sequences conferring substrate specificity are indicated by bold letters. The ribozyme cleavage site, at nucleotide position 955 (Goldberg, G.I. et al., 1986), is indicated by an arrow.

Figure 3

Cleavage of Interstitial Collagenase RNA by a "Hammerhead" Ribozyme ICRZ1

Autoradiograph of ribozyme cleavage of a short substrate corresponding to a portion of the interstitial collagenase mRNA. The 0mM Mg^{++} lane represents the control reaction in which no Mg^{++} has been added and the 30mM Mg^{++} lane represents the reaction in which 30mM Mg^{++} is added to elicit ribozyme cleavage. "RZ" represents ribozyme, "S" denotes substrate and "CP" indicates cleavage products.

Figure 4

Ran/TC4 mRNA-specific "Hammerhead" Ribozymes

Three "hammerhead" ribozymes targeted to different cleavage sites of the Ran/TC4 mRNA are shown. Partial sequences of the substrate RNA are indicated by italics. Ribozyme guide sequences conferring substrate specificity are indicated by bold letters. The ribozyme cleavage site is indicated by an arrow. TC4RZ1 cleaves the substrate RNA at nucleotide position 83, TC4RZ2 cleaves at position 102, and TC4RZ3 cleaves at position 223. All nucleotide positions reflect the numbering according to Drivas et al. (1990) but also include four additional nucleotides derived from the transcribed vector sequence.

Figure 5

Cleavage of Ran/TC4 RNA by "Hammerhead" Ribozymes

Autoradiograph of three sets of ribozyme cleavage reactions. The unlabeled ribozymes, TC4RZ1, TC4RZ2, and TC4RZ3, are each targeted to a different site on the labeled Ran/TC4 RNA. In each

reaction set, the 0mM Mg^{++} lane represents the control reaction in which no Mg^{++} has been added, and the 30mM Mg^{++} lane represents the reaction in which 30mM Mg^{++} is added to elicit ribozyme cleavage. "S" denotes substrate and "CP" indicates cleavage products.

Figure 6

Interleukin-6 mRNA-specific "Hammerhead" Ribozyme

A "hammerhead" ribozyme (IL6RZ1) and target substrate (interleukin-6 RNA) complex is shown. Partial sequence of the substrate RNA is indicated by italics. Ribozyme guide sequences conferring substrate specificity are indicated by bold letters. The ribozyme cleavage site, at nucleotide position 510 (May, L.T. et al., 1986), is indicated by an arrow.

Figure 7

Cleavage of Interleukin-6 RNA by a "Hammerhead" Ribozyme IL6RZ1

Autoradiograph of ribozyme cleavage of a short substrate corresponding to a portion of the interleukin-6 mRNA. The 0mM Mg^{++} lane represents the control reaction in which no Mg^{++} has been added and the 30mM Mg^{++} lane represents the reaction in which 30mM Mg^{++} is added to elicit ribozyme cleavage. "RZ" represents ribozyme, "S" denotes substrate and "CP" indicates cleavage products.

Figure 8

5-Alpha-Reductase mRNA-specific "Hammerhead" Ribozyme

A "hammerhead" ribozyme (5ARRZ1) and target substrate (5-alpha-reductase RNA) complex is shown. Partial sequence of the substrate RNA is indicated by italics. Ribozyme guide sequences conferring substrate specificity are indicated by bold letters. The ribozyme cleavage site, at nucleotide position 656 (Andersson, S. et al., 1991), is indicated by an arrow.

Figure 9

Cleavage of 5-Alpha-Reductase RNA by a "Hammerhead" Ribozyme 5ARRZ1

Autoradiograph of ribozyme cleavage of a short substrate corresponding to a portion of the 5-alpha-reductase mRNA. The 0mM Mg^{++} lane represents the control reaction in which no Mg^{++} has been added and the 30mM Mg^{++} lane represents the reaction in which 30mM Mg^{++} is added to elicit ribozyme cleavage. "RZ" represents ribozyme, "S" denotes substrate and "CP" indicates cleavage products.

DETAILED DESCRIPTION OF THE INVENTION

Ribozyme Preparation and Application.

The site of cleavage in a target molecule RNA is dependent on the selection of the ribozyme. For example, if the ribozyme is of the hammerhead type (see Figure 1 and following examples), the substrate cleavage site is immediately 3' to the sequence NUX, where N is any nucleotide, X is any nucleotide except G, and U is uridine. The length of complementary sequence between ribozyme and target will vary with each ribozyme system and the particular therapeutic setting or application. In the case of hammerhead ribozymes, the length of each hybridizing arm (stems 1 and 3 in Figure 1) is generally 5-15 residues and results in base pairing to the substrate sequences immediately upstream and downstream of the UX sequence of the cleavage site. It is clear that several different types of ribozymes may be employed to achieve the specific cleavage of the targeted RNA molecules delineated here.

Appropriate oligonucleotides are synthesized enzymatically by conventional techniques which are well known to those skilled in the art, or by using standard solid phase synthesis techniques using a commercially available machine such as an Applied Biosystems Incorporated, Model 392 DNA/RNA synthesizer. Oligonucleotides can also be synthesized by solution phase methods or recombinant DNA methods known to those skilled in the art.

The ribozyme oligonucleotides of the invention may be conventional single-stranded RNA molecules or they may be partially or wholly modified by introducing modifications of the

backbone (e.g. mono- and dithio esters, methylphosphonates); modifications of the sugar moiety (e.g. deoxyribose, 2' alkyl groups, arabinose, alpha-anomers); and modifications of the base (e.g. 2-aminopurine, inosine) using techniques and methods known to those skilled in the art. See for example: Usman, N. and Cedergren, R., 1992; Cook, P.D., 1991; Heidenreich, O. et al., 1993; Morvan, F. et al., 1991, the contents of which are hereby incorporated by reference in their entirety. These oligonucleotides may be further modified by the conjugation of chemical moieties either internally or terminally, such as: polycations (e.g. poly(L)lysine); hydrophobic groups (e.g. cholesterol); oligopeptides (e.g. Arg-Gly-Asp-Ser); or polypeptides (e.g antibodies) using techniques and methods known to those skilled in the art (see for example: Lippert, B., 1992; Clarenc, J.P. et al., 1993; Bunnell, B.A. et al., 1992; Kuijpers, W.H. et al., 1993; Citro, G. et al., 1992; Leamon, C.P. and Low. P.S., 1991, the contents of which are hereby incorporated by reference in their entirety).

The above modifications are designed to increase the stability, activity, or cellular uptake and transport of the ribozymes. It is obvious that many other modifications can be introduced into these oligonucleotides to improve their overall performance without departing from the spirit and scope of this invention.

Delivery of Oligonucleotides to Cutaneous Targets.

As with all therapeutics, the efficiency of delivery to a defined site can affect ultimate clinical efficacy . Ribozyme molecules have to reach the cell surface, cross the cell membrane, and act, either in the cytoplasm or the nucleus, on the target RNA. In general, however, the delivery of nucleic acid molecules to the skin is easier than to other tissues or organs in that the composition comprising the ribozyme is applied directly on a small exposed area. A high concentration of the therapeutic can be administered with little systemic side effects.

A potential obstacle for the delivery of nucleic acid molecules to cutaneous targets is the stratum corneum (SC). The stratum corneum is a matrix of keratinized cells, surrounded by lipids including cholesterol, free fatty acids and ceramides. These lipids form multilamellar arrays which impart resistance to percutaneous flux of polar compounds. However, the lamellar sheets are not conjoined and gaps between the sheets allow the passage of polar compounds to the lower epidermis and dermis. Similar openings also exist in the sheaths of hair follicles. In contrast, non-polar compounds, can easily diffuse through the SC because they readily partition in the lipid phase. These properties of the skin allow one to approach cutaneous delivery of nucleic acid molecules in two ways: Direct application of high concentrations, or by alteration of overall hydrophobicity. Each of these approaches may involve several specific protocols, such as those listed below:

1. Modification of the nucleic acid with lipophilic moieties such as alkylphosphonates and methyl or ethyl groups (described above).
2. Covalent attachment of lipids such as triglycerides or cholesterol (described above).
3. Use of emulsions and lipophilic compounds, as they are generally known in the art, to enhance dermal penetration.
4. Use of enhancers as they are generally known in the art (such as N-dodecyl di-isopranolamine and oleyl alcohol) to improve transport.
5. Use of iontophoresis (electrotransport), as it is generally known in the art, to introduce charged oligonucleotides through hair follicles and sweat glands into the skin.
6. Use of electroporation, as it is generally known in the art, to induce changes in the underlying skin that will allow the passage of nucleic acid molecules into the dermis.
7. Use of liposomes to carry nucleic acid molecules to targeted substrates. The liposomal formulation can be

either cationic, where the oligonucleotides bind to the exterior of the liposome, or conventional, where the nucleic acid molecules are entrapped within the vesicles. Both liposomal formulations will enhance the crossing of the SC barrier. In addition, liposomes are readily taken up by cells (keratinocytes, melanocytes, fibroblasts, and cells of immune origin) within which they can release part or most of their therapeutic contents. Liposomes are widely used for delivery in cosmetic and topical applications, and applicable techniques and formulations are well known to those skilled in the art.

8. Conjugation of receptor-specific ligands directly to nucleic acid molecules to enhance their uptake by specific cell populations. Alternatively, liposomes can be tagged with these ligands for receptor mediated entry into the cell. The techniques for coupling specific ligands will depend on the particular ligand and the cell type one wishes to target, but are well known to those skilled in the art.

9. Use of replicating or non-replicating RNA or DNA vectors encoding ribozyme sequences. A large variety of such vectors are available or can be engineered using common molecular biological techniques well known to those skilled in the art.

In addition, the formulations of vehicles (e.g. ointments, gels, creams) commonly used in topical applications are generally known and may also be used with the inventive oligonucleotides. Some of the methods listed above can be found in: Zhu, N. et al., 1993; Juliano, R.L. and Akhtar, S., 1992; Chavany, C. et al., 1992; L'Huillier, P.J. et al., 1992; Hsieh, S.Y. and Taylor, 1992; Citro, G. et al., 1992; Ropert, C. et al., 1992; Sioud, M. et al., 1992; Hori, M. et al., 1989; Gao, X. and Huang, L., 1991; Malone, R.W. et al., 1989, the contents of which are hereby incorporated by reference in their entirety.

Sequences of Therapeutic Ribozymes for Specific Cutaneous Conditions.

The invention provides a number of ribozyme sequences, set forth in Table I below, designed to treat specific cutaneous conditions. The names of the hammerhead ribozymes that are used in the accompanying examples are shown in column A. The nucleotide sequences of the ribozymes are shown in column B. The consensus catalytic core of the hammerhead ribozyme, 5' CUG-AUG-AGU-CCG-UGA-GGA-CGA-AA 3', is italicized and underlined in each ribozyme sequence. The RNA targets against which specific ribozymes are designed are shown in column C. These targets and the cutaneous conditions they are associated with, are described more fully herein. Column D lists the ribozyme cleavage site in the specified target RNA. Cleavage takes place 3' to the third nucleotide of the triplet listed. The location of the cleavage site in the target RNA is indicated by the number next to the third nucleotide and corresponds to the nucleotide position in the published sequence (see specific examples). Column E indicates the example number where the utilization of the specific ribozyme is described.

Table I

(A)	(B)	(C)	(D)	(E)
Ribozyme	Ribozyme Sequence (5' to 3')	Target RNA	Cleavage site	Example #
ICRZ1	CUGUCUUUAAAGCUGAUGAGUCCGUGAGGACGAAACAUCACUGC	Collagenase	GUC(395)	1
EL1RZ1	GCUCCCAGCUGAUGAGUCCGUGAGGACGAAACAGCCC	Elastase	GUC(287)	2
EL2RZ1	AUCUUAGACUGAUGAGUCCGUGAGGACGAAAACUGAC	Elastase 2	GUC(237)	3
ELNRZ1	AAGGCCCCCUGAUGAGUCCGUGAGGACGAAACCCUGC	Elastin	CUC(236)	4
AATRZ1	UGCUGUUGCUGAUGAGUCCGUGAGGACGAAAUGGUGU	Alpha1-antitrypsin	GUC(344)	5
CDRZ1	GCUGGACACUGAUGAGUCCGUGAGGACGAAAGGAGUU	CD44	AUC(314)	6
TC4RZ1	CCAACCAACUGAUGAGUCCGUGAGGACGAAACAACUC	Kaposi/TC4	GUA(40)	7
TC4RZ2	UUUUCCAGCUGAUGAGUCCGUGAGGACGAAACCACCA	Kaposi/TC4	GUA(107)	7
TC4RZ3	AUACAUUGCUGAUGAGUCCGUGAGGACGAAACUGAAC	Kaposi/TC4	GUC(222)	7
TGFRZ1	GCAUCAAACUGAUGAGUCCGUGAGGACGAAACUGCAC	TGF-alpha	GUC(217)	8
TNFRZ1	ACAUGGGCCUGAUGAGUCCGUGAGGACGAAAGGCUC	TNF-alpha	GUA(162)	9
IL6RZ1	GAACUGGAUCAGCUGAUGAGUCCGUGAGGACGAAAUCUCCUGUCAC	IL-6	GUC(516)	10
IL1ARZ1	UGCCUUGCCUGAUGAGUCCGUGAGGACGAAAUACCAC	IL-1-alpha	GUC(327)	11
IL1BRZ1	CAACAACUCUGAUGAGUCCGUGAGGACGAAAGCGUGC	IL-1-beta	GUC(230)	12
ILRZ1	GCUGCCAACUGAUGAGUCCGUGAGGACGAAAAGCCAC	IL-1	CUC(1410)	13
ICAMRZ1	GCAGGAGCCUGAUGAGUCCGUGAGGACGAAACCCUUC	ICAM-1	GUC(521)	14
SARRZ1	AGUGCUGCGAGGCUGAUGAGUCCGUGAGGACGAAAGCUAGUGGC	Sarcoma-associated gene	GUC(644)	15
TYRRZ1	AAGGCCAACUGAUGAGUCCGUGAGGACGAAAUCCCGG	Tyrosinase	GUC(1222)	16
GRBRZ1	ACACAUCCCUGAUGAGUCCGUGAGGACGAAAAUUGAC	Growth factor receptor	GUC(788)	17

Cutaneous Disorders Amenable to Nucleic Acid-Based Therapeutics.

Aging.

Skin aging can be classified into two types--intrinsic (chronologic) aging and actinic (photo) aging. Intrinsic aging involves those alterations occurring in the skin of older individuals which has been protected from the sun. It is manifested cosmetically as fine wrinkling. Actinic aging is the result of long-term exposure of skin to sunlight and is characterized by deep wrinkling, furrowing, and drying. The majority of undesirable features associated with aging skin are the result of UV-induced photoaging (Uitto, J. et al., 1989b; Klingman, A.M., 1969). Especially important in relation to the present invention is that the histologic changes associated with photoaged skin have been demonstrated to be reversible (Gilhar, A. et al., 1991).

Treatments for aged skin, essentially designed to reduce wrinkling, have included cosmetic plasty, chemical peels to promote collagen formation, and the implantation of silicone or collagen. Pharmacological treatments have included anti-inflammatory agents such as hydrocortisone and, most recently, various natural and synthetic members of the vitamin A family, the retinoids (even though the latter may exhibit acute toxicities and complications of long-term usage).

A major aspect of both intrinsic and actinic aging includes a decrease in the rate of keratinocyte, melanocyte, and fibroblast cell turnover. Studies done on intrinsically aged skin fibroblasts and keratinocytes have demonstrated a marked decline in their growth potential and responsiveness to mitogens, as well as an increased responsiveness to growth inhibitors (Plinsko, A. and Gilchrest B.A., 1983; Stanulis-Praeger, B.M. and Gilchrest, B.A., 1986; Amoto, T.K. et al., 1989). Of particular interest is an interferon-like molecule in the basal layer of the epidermis that can serve as a modulator inhibiting cellular proliferation (Yaar, M. et al, 1986), and the fact that interferons have been shown to have a pronounced growth inhibitory effect on aged keratinocytes (Yaar, M. et al, 1989; Peacoeke, M. et al., 1989).

Such proliferation inhibitors are good targets for intervention to reverse age related cutaneous symptoms. Ribozyme molecules can be designed which will disrupt the synthesis of interferons alpha, beta, and gamma (Oliver, G. et al., 1985; Fiers, W. et al., 1982; Nishi, T. et al., 1985). Restoration of the proliferative and synthetic capacity of epidermal and dermal cells should reverse at least some of the cosmetic effects of aging.

Numerous studies have also indicated that there is an alteration in the content of the major structural components of the dermal extracellular matrix (ECM) in aged skin. The production of type I collagen, which plays the major role in providing tensile strength, has been shown to decrease in both actinically and intrinsically aged skin (Uitto, J., 1989; Uitto, J., et al., 1989a; Warren, R. et al., 1991). In UV-irradiated skin cell models, elevated levels of interstitial collagenase (the enzyme responsible for the breakdown of dermal collagen) have been detected, contributing to the loss of collagen content (Mammone, T. et al., 1992; Petersen, M.J. and Li, H.L., 1992).

All-trans retinoic acid (tretinoin) has been shown to be effective in reversing many symptoms associated with actinic aging, both by inhibiting collagenase production and by stimulating collagen synthesis (Schwartz, E. et al., 1991; Chen, S. et al., 1992; Kligman, L.H., 1989; Uitto, J. et al., 1988b).

Taken together, these studies indicate that an increase in the collagen content of aged skin, achieved by inhibition of interstitial collagenase, could result in wrinkle effacement and reversal of aged appearance. Ribozyme molecules (see Example 1 and Figure 2) can be designed which will disrupt the synthesis of the collagenase enzyme.

Collagenase inhibition has other potential uses apart from the reversal of cutaneous aging. It can also be used in certain forms of arthritis where there is an increased degradation of collagen, and also in certain inherited disorders of collagen metabolism. In genetically inherited diseases such as dystrophic epidermolysis bullosa and Werner's syndrome, where the skin resembles that of aged individuals, elevated levels of

collagenase have been observed (Unemori, E.N. et al., 1992, Korenfeld, C.D. et al., 1992; Altman, J. et al., 1992). Furthermore, this therapeutic should be useful in hastening the process of wound healing by increasing the matrix of the regenerating skin.

Studies done on intrinsically aged skin and observations made with skin from patients having heritable diseases of connective tissue in which elastic fibers are severely affected, such as cutis laxa or pseudoxanthoma elasticum (Uitto, J. et al., 1988a), suggest that elastic fibers are responsible for elasticity and resilience (Uitto, J., 1986). Cell culture studies have demonstrated a steady decrease in elastin mRNA after age 30 (Fazio, M.J. et al., 1988), and low levels of elastin production in fibroblasts of individuals aged 70 years and older (Sephel, G. et al., 1987). The loss of functional elastic fibers may therefore explain some of the symptoms of intrinsic aging, such as wrinkling and sagging.

The enzymes that degrade elastin (elastases) provide valuable targets for therapeutic intervention. Inhibition of these enzymes should result in an increase in functional elastin content thereby reversing some of the symptoms of intrinsic aging. Ribozyme molecules can be used to reduce the synthesis of the principal elastases produced by fibroblasts and by polymorphonuclear leukocytes (see Table I and Examples 2 and 3).

These therapeutics will have more than cosmetic value. They can also be used in several diseases where functional elastin fiber content is altered, such as emphysema. They might also find use in general connective tissue weakness of arteries where the drug can be applied by a systemic route.

In addition to interstitial collagenase and elastases, there are several other metalloproteinases which are partly responsible for the breakdown of ECM fibers--neutrophil collagenase (Hasty, K.A. et al., 1990), type IV collagenase (Wilhelm, S.M. et al., 1989), and stromelysins 1 and 2 (Whitman, S.E. et al., 1986; Muller, D. et al., 1988). Inhibiting production of these enzymes with ribozymes should also prove beneficial for the repair of the ECM.

The hallmark of actinic aging is dermal elastosis--the accumulation of elastotic material and the distortion of normal elastic fiber architecture. The exact biochemical composition of this material is not known. Inhibition of elastin synthesis by ribozymes directed against elastin mRNA should result in a decrease in the dermal elastin content (Table I and Example 4). Other targets for modulating elastin levels are the proteins that inhibit elastases, such as alpha-1-antitrypsin (Table I and Example 5) and alpha-2-macroglobulin (Kan, C.C-. et al., 1985). Inhibition of the production of these proteins by ribozyme molecules should ultimately result in decreased elastin levels.

There are some indications that the levels of glycosaminoglycans and proteoglycans decrease with intrinsic aging (Johnston, K.J. et al., 1985). It is likely that an increased amount of glycosaminoglycans in the ECM, especially hyaluronic acid, will result in increased hydration and maintenance of turgor, giving aged skin a fuller and younger look. This can be achieved by ribozyme inhibition of the extracellular enzyme hyaluronidase, which is responsible for the breakdown of hyaluronic acid and other glycosaminoglycans (Bertolami, C.N. and Donoff, R.B., 1982). These ribozymes can also be used in any disorders where there is a decreased concentration of glycosaminoglycans, e.g. wound healing, where glycosaminoglycans have been shown to have a beneficial role. Since some glycosaminoglycans are degraded in fibroblasts, ribozyme molecules can also be developed that will inhibit the synthesis of hyaluronic acid-binding proteins present on the surfaces of dermal cells. These binding proteins include CD44 (Laurent, T.C. and Fraser J.R., 1992). The inhibition of its synthesis by ribozymes should result in an increase in the ECM concentration of hyaluronic acid (Table I and Example 6). These ribozymes may also be of therapeutic value in the control of tumorigenesis and metastasis, where interactions of tumor cells with components of the ECM affect the migration of these cells.

The present invention will be further illustrated by reference to the following non-limiting examples:

Example 1: Ribozyme cleavage of a short sequence corresponding to a portion of the interstitial collagenase mRNA, a therapeutic target in the treatment of intrinsic and actinic cutaneous aging.

A fragment of human interstitial collagenase antisense DNA (nucleotides 910-1009; Goldberg, G.I. et al., 1986; GenBank accession #M13509) attached to the T7 RNA polymerase promoter sequence was synthesized on a ABI synthesizer. ^{32}P labeled RNA transcripts (100 nucleotides in length), corresponding to a portion of human interstitial collagenase mRNA were generated essentially as described by Milligan et al., (1987), the content of which is hereby incorporated by reference in its entirety. A labeled ribozyme ICRZ1 (sequence composition 5'-CUG-UCU-UUA-AAG-CUG-AUG-AGU-CCG-UGA-GGA-CGA-AAC-AUC-ACU-UC-3', see Figure 2), designed to cleave after nucleotide 955, was also prepared according to Milligan et al., (1987), using a synthetic deoxyoligonucleotide template (5'GAA-GTG-ATG-TTT-CGT-CCT-CAC-GGA-CTC-ATC-AGC-TTT-AAA-GAC-AGC-TAT-AGT-GAG-TCG-TAT-TA 3') and a short synthetic deoxyoligonucleotide-"18mer" (5' TAA-TAC-GAC-TCA-CTA-TAG 3').

Briefly, each RNA was transcribed in a 100ul (ul=microliter) reaction mixture containing 4ul of 1M Tris-HCl, pH 7.6, 2.4ul of 1M $MgCl_2$, 2ul 100mM spermidine, 1ul of 1M DTT, 10ul of 0.1% Triton X-100, 4ul each of 100mM GTP, ATP, CTP, and UTP, 10ul of 800mg/ml PEG, 1ul of RNasin RNase inhibitor (40units/ul) (Promega Corporation, Madison, WI), 2ul of alpha-^{32}P CTP (800Ci/mmol) (NEN Research Products, Boston, MA), 5ul of T7 RNA polymerase (300 units/ul) (New England Biolabs, Beverly, MA), 4ul of 5uM template DNA, 4ul of 5uM 18mer DNA, and 37.6ul H_2O. Both ribozyme and substrate were separated on a 15% 7M urea/polyacrylamide gel, identified as the predominant band by U.V. shadowing, eluted and precipitated.

For cleavage reactions, the ribozyme and the substrate were mixed in a 1:2 molar ratio in a final volume of 10ul containing 50mM Tris-HCl (pH 7.6) either in the presence or absence of 30mM

$MgCl_2$. The reactions were incubated at 37 C for 90 minutes, terminated with the addition of an equal volume of loading dye (0.25% bromophenol blue, 0.25% xylene cyanol, 50% formamide, and 30mM EDTA), heated to 90 C for 2 minutes, resolved on a 15% 7M urea/polyacrylamide gel, and autoradiographed. As shown in Figure 3, when the ribozyme ICRZ1 and target collagenase RNA substrate were incubated in the presence of 30mM Mg^{++}, the ICRZ1 ribozyme effectively cleaved collagenase RNA producing two cleavage products of the expected size of 46 nucleotides (nts) and 54nts in length.

Based on the demonstrated activity of the above ICRZ1 ribozyme composition, it may be used to cleave collagenase mRNA both *in vitro* and *in vivo*. A therapeutically effective amount of this ribozyme composition (10ng to 1mg) can be incorporated into a formulation consisting of a delivery vehicle or carrier such as cationic liposomes. This formulation may be applied directly to an affected area of the skin. The ribozyme composition contained in this formulation may be used to cleave the collagenase mRNA present in skin cells and thus it may modify the expression of the target protein and may thereby mitigate the cutaneous condition.

Example 2: **Ribozyme cleavage of a short sequence corresponding to a portion of the elastase/medullasin mRNA, a therapeutic target in the treatment of intrinsic cutaneous aging.**

A fragment of human elastase/medullasin antisense DNA (nucleotides 242-322, Okano, K. et al., 1989; GenBank accession #M34379) attached to the T7 RNA polymerase promoter sequence can be synthesized and ^{32}P labeled RNA transcripts (81 nucleotides in length), corresponding to a portion of human elastase/medullasin mRNA may be generated. Labeled ribozyme ELMRZ1 (sequence composition 5'-GCU-CCC-AGC-UGA-UGA-GUC-CGU-GAG-GAC-GAA-ACA-CCC-G-3', see Table I), designed to cleave after nucleotide 287, can also be prepared using a synthetic deoxyoligonucleotide template (5' CGG-GTG-TTT-CGT-CCT-CAC-GGA-CTC-ATC-AGC-TGG-GAG-CC-TAT-AGT-GAG-TCG-TAT-TA 3') and a short synthetic deoxyoligonucleotide-

"18mer" (5' TAA-TAC-GAC-TCA-CTA-TAG 3'), according to the methods of Example 1. For cleavage reactions, the ribozyme ELMRZ1 and the elastase/medullasin RNA substrate can be mixed in a 1:2 molar ratio, and incubated at 37°C for 90 minutes. When ribozyme and target substrate are incubated in the presence of 30mM Mg^{++}, two cleavage products of the expected sizes of 35nts and 46nts should be produced.

The ELMRZ1 ribozyme composition may be used to cleave elastase/medullasin mRNA both *in vitro* and *in vivo*. A therapeutically effective amount of this ribozyme composition (10ng to 1mg) can be incorporated into a formulation consisting of a delivery vehicle or carrier such as cationic liposomes. This formulation may be applied directly to an affected area of the skin. The ribozyme composition contained in this formulation may be used to cleave the elastase/medullasin mRNA present in skin cells and thus it may modify the expression of the target protein and may thereby mitigate the cutaneous condition.

Example 3: **Ribozyme cleavage of a short sequence corresponding to a portion of the elastase 2 mRNA, a therapeutic target in the treatment of intrinsic cutaneous aging.**

A fragment of human elastase 2 antisense DNA (nucleotides 282-362, Fletcher, T.S. et al., 1987; GenBank accession #M16631) attached to the T7 RNA polymerase promoter sequence can be synthesized and ^{32}P labeled RNA transcripts (81 nucleotides in length), corresponding to a portion of human elastase 2 mRNA may be generated. Labeled ribozyme EL2RZ1 (sequence composition 5'-AUC-UUA-GAC-UGA-UGA-GUC-CGU-GAG-GAC-GAA-AAC-UGA-C-3', see Table I), designed to cleave after nucleotide 327, can also be prepared using a synthetic deoxyoligonucleotide template (5' GTC-AGT-TTT-CGT-CCT-CAC-GGA-CTC-ATC-AGT-CTA-AGA-TC-TAT-AGT-GAG-TCG-TAT-TA 3') and a short synthetic deoxyoligonucleotide-"18mer" (5' TAA-TAC-GAC-TCA-CTA-TAG 3') according to the methods of Example 1. For cleavage reactions, the ribozyme EL2RZ1 and the elastase 2 RNA substrate can be mixed in a 1:2 molar ratio, and incubated at 37°C for 90 minutes. When ribozyme and target substrate are

incubated in the presence of 30mM Mg^{++}, two cleavage products of the expected sizes of 35nts and 46nts should be produced.

The EL2RZ1 ribozyme composition may be used to cleave elastase 2 mRNA both *in vitro* and *in vivo*. A therapeutically effective amount of this ribozyme composition (10ng to 1mg) can be incorporated into a formulation consisting of a delivery vehicle or carrier such as cationic liposomes. This formulation may be applied directly to an affected area of the skin. The ribozyme composition contained in this formulation may be used to cleave the elastase 2 mRNA present in skin cells and thus it may modify the expression of the target protein and may thereby mitigate the cutaneous condition.

Example 4: Ribozyme cleavage of a short sequence corresponding to a portion of the elastin mRNA, a therapeutic target in the treatment of actinic cutaneous aging.

A fragment of human elastin antisense DNA (nucleotides 241-321, Fazio, M.J. et al., 1988a; GenBank accession #M36860) attached to the T7 RNA polymerase promoter sequence can be synthesized and ^{32}P labeled RNA transcripts (81 nucleotides in length), corresponding to a portion of human elastin mRNA may be generated. Labeled ribozyme ELNRZ1 (sequence composition 5'-AAG-GCG-CCC-UGA-UGA-GUC-CGU-GAG-GAC-GAA-ACC-CUG-C-3', see Table I), designed to cleave after nucleotide 286, can also be prepared using a synthetic deoxyoligonucleotide template (5' GCA-GGG-TTT-CGT-CCT-CAC-GGA-CTC-ATC-AGG-GCG-CCT-TC-TAT-AGT-GAG-TCG-TAT-TA 3') and a short synthetic deoxyoligonucleotide-"18mer" (5' TAA-TAC-GAC-TCA-CTA-TAG 3') using the methods of Example 1. For cleavage reactions, the ribozyme ELNRZ1 and the elastin RNA substrate can be mixed in a 1:2 molar ratio, and incubated at 37°C for 90 minutes. When ribozyme and target substrate are incubated in the presence of 30mM Mg^{++}, two cleavage products of the expected sizes of 35nts and 46nts should be produced.

The ELNRZ1 ribozyme composition may be used to cleave elastin mRNA both *in vitro* and *in vivo*. A therapeutically effective amount of this ribozyme composition (10ng to 1mg) can

be incorporated into a formulation consisting of a delivery vehicle or carrier such as cationic liposomes. This formulation may be applied directly to an affected area of the skin. The ribozyme composition contained in this formulation may be used to cleave the elastin mRNA present in skin cells and thus it may modify the expression of the target protein and may thereby mitigate the cutaneous condition.

Example 5: Ribozyme cleavage of a short sequence corresponding to a portion of the alpha-1-antitrypsin mRNA, a therapeutic target in the treatment of actinic cutaneous aging.

A fragment of human alpha-1-antitrypsin antisense DNA (nucleotides 199-279, Colau, B. et al., 1984; GenBank accession #K01396) attached to the T7 RNA polymerase promoter sequence can be synthesized and ^{32}P labeled RNA transcripts (81 nucleotides in length), corresponding to a portion of human alpha-1-antitrypsin mRNA may be generated. Labeled ribozyme AATRZ1 (sequence composition 5'-UGC-UGU-UGC-UGA-UGA-GUC-CGU-GAG-GAC-GAA-AUG-GUG-U-3', see Table I), designed to cleave after nucleotide 244, can also be prepared using a synthetic deoxyoligonucleotide template (5' ACA-CCA-TTT-CGT-CCT-CAC-GGA-CTC-ATC-AGC-AAC-AGC-AC-TAT-AGT-GAG-TCG-TAT-TA 3') and a short synthetic deoxyoligonucleotide-"18mer" (5' TAA-TAC-GAC-TCA-CTA-TAG 3'), according to the methods of Example 1. For cleavage reactions, the AATRZ1 ribozyme and the alpha-1-antitrypsin RNA substrate can be mixed in a 1:2 molar ratio, and incubated at 37°C for 90 minutes. When ribozyme and target substrate are incubated in the presence of 30mM Mg^{++}, two cleavage products of the expected sizes of 35nts and 46nts should be produced.

The AATRZ1 ribozyme composition may be used to cleave alpha-1-antitrypsin mRNA both *in vitro* and *in vivo*. A therapeutically effective amount of this ribozyme composition (10ng to 1mg) can be incorporated into a formulation consisting of a delivery vehicle or carrier such as cationic liposomes. This formulation may be applied directly to an affected area of the skin. The ribozyme composition contained in this formulation may be used to cleave the alpha-1-antitrypsin mRNA present in skin cells and

thus it may modify the expression of the target protein and may thereby mitigate the cutaneous condition.

Example 6: Ribozyme cleavage of a short sequence corresponding to a portion of the CD44 mRNA, a therapeutic target in the treatment of intrinsic and actinic cutaneous aging.

A fragment of human CD44 antisense DNA (nucleotides 271-351, Harn, H.J. et al., 1991; GenBank accession #M59040) attached to the T7 RNA polymerase promoter sequence will be synthesized and ^{32}P labeled RNA transcripts (81 nucleotides in length), corresponding to a portion of human CD44 mRNA will be generated. Labeled ribozyme CDRZ1 (sequence composition 5'-GCU-GCA-CAC-UGA-UGA-GUC-CGU-GAG-GAC-GAA-AGG-AGU-U-3', see Table I), designed to cleave after nucleotide 316 will also be prepared using a synthetic deoxyoligonucleotide template (5' AAC-TCC-TTT-CGT-CCT-CAC-GGA-CTC-ATC-AGT-GTG-CAG-CC-TAT-AGT-GAG-TCG-TAT-TA 3') and a short synthetic deoxyoligonucleotide-"18mer" (5' TAA-TAC-GAC-TCA-CTA-TAG 3'), according to the methods of Example 1. For cleavage reactions, the CDRZ1 ribozyme and the CD44 RNA substrate will be mixed in a 1:2 molar ratio, and be incubated at 37°C for 90 minutes. When ribozyme and target substrate are incubated in the presence of 30mM Mg^{++}, two cleavage products of the expected size of 35nts and 46nts in length should be produced.

The CDRZ1 ribozyme composition may be used to cleave CD44 mRNA both *in vitro* and *in vivo*. A therapeutically effective amount of this ribozyme composition (10ng to 1mg) can be incorporated into a formulation consisting of a delivery vehicle or carrier such as cationic liposomes. This formulation may be applied directly to an affected area of the skin. The ribozyme composition contained in this formulation may be used to cleave the CD44 mRNA present in skin cells and thus it may modify the expression of the target protein and may thereby mitigate the cutaneous condition.

Psoriasis.

Psoriasis vulgaris is a chronic skin disorder affecting between 1% and 3% of the population. It is characterized by inflammation and by epidermal hyperproliferation. A number of therapeutic approaches are currently employed against psoriasis. Anti-proliferative agents are anthralin, methotrexate, and psoralens plus UVA radiation (320-400 nm) ("PUVA"); anti-inflammatory agents are cyclosporin, corticosteroids, and vitamin D, and agents whose functions are unclear are retinoids. While topical therapies such as anthralin and corticosteroids appear to be of only moderate success for limited psoriasis, the value of systemic therapies such as retinoids and PUVA for more severe, recalcitrant psoriasis, where clinical responses are good, is complicated by toxicity. All of these drugs have associated complications (Ellis, C.N. et al., 1987; Stern, R.S. and Lange, R.J., 1988; Goldsmith, P. et al., 1989).

Although the etiology of psoriasis is unknown, listed below are molecules implicated in psoriatic pathophysiology whose modulation by ribozymes should result in the relief, if not the reversal of its symptoms.

Ran/TC4.

Human Ran/TC4 is a small nuclear GTP binding and hydrolyzing protein (GTPase)(Dasso, M., 1993) which functions as part of a mitotic checkpoint control that prevents the onset of mitosis until the completion of DNA synthesis. The loss of Ran/TC4 results in a fatal premature initiation of mitosis. In relation to the hyperproliferation of keratinocytes in psoriasis, the use of ribozymes to reduce Ran/TC4 expression (Figure 4 and Example 7) may be of particular value for enhancing the efficacy and greatly reducing the toxicity of currently used therapeutics, such as methotrexate. Specifically, reduction in the Ran/TC4 level may in itself induce cell death in the proliferating cell population. In addition, in the presence of low levels of methotrexate that are not as toxic to normal cells (levels well below those now being used to treat psoriasis), hyperproliferating cells deficient in Ran/TC4 will remain sensitive. In other words, the partial DNA synthesis inhibition

induced by methotrexate will not prevent psoriatic cells lacking Ran/TC4 from undergoing premature mitosis and eventual death.

Epidermal Growth Factor (EGF) receptors, Transforming Growth Factor (TGF)-alpha and Tumor Necrosis Factor (TNF)-alpha.

Several hyperproliferative diseases including psoriasis are characterized by the overexpression and altered distribution of EGF receptors and TGF-alpha (Elder, J.T. et al., 1989; Watts, P. et al., 1990; Detmar, M. et al., 1992). In psoriasis they contribute in an autocrine fashion to the hyperproliferation of keratinocytes (Watts, J. et al., 1992). TNF-alpha has also been found in increased levels in psoriatic lesions relative to those of normal skin, and mRNA and protein levels of this cytokine were significantly decreased in peripheral blood mononuclear cells of psoriatic patients after PUVA therapy (Neuner, P. et al., 1992). It appears that this cytokine can upregulate TGF-alpha and growth factor receptors, triggering cellular hyperproliferation. Ribozyme molecules can be designed which will disrupt the synthesis of EGF receptors (Ullrich, A. et al., 1984), TGF-alpha (Table I and Example 8) and TNF-alpha (Table I and Example 9).

Interleukin (IL)-6.

Several lines of evidence indicate the involvement of IL-6 in psoriasis, including a 3-fold increase in the level of IL-6 secreted by cultured fibroblasts from psoriatic lesions compared to that of cells from normal skin (Debets, R. et al., 1992) and immunohistochemical staining of psoriatic keratinocytes revealing intense labeling of IL-6 in the cytoplasm close to the membrane (Castells-Rodellas, A., 1992). IL-6 is thus an attractive target for intervention. Ribozyme molecules designed against IL-6 mRNA should lower IL-6 levels (see Figure 6 and Example 10). Such anti-IL-6 therapeutics will have uses beyond the treatment of psoriasis, since IL-6 has also been implicated in the manifestations of many immune response diseases and bacterial infections.

Interleukin (IL) 1 and IL-8.

IL-8 was consistently found to be elevated in tissue extracts of lesional psoriatic skin, compared to the uninvolved skin of the same patient (Takematsu, H. and Tagami, H., 1993). Supernatants from lesional psoriatic cell culture potentiated the acute activation of T-lymphocytes, a step apparently critical for the maintenance of the lesions (Chang, E.Y. et al., 1992). This potentiation was directly correlated with the severity of the lesion. Anti-IL-1 and IL-8 antibodies partly blocked the potentiation, suggesting that IL-1 and IL-8 are partly responsible for lesion maintenance. In addition, IL-1 and IL-8 levels have been shown to decrease in patients receiving PUVA therapy (Neuner, P. et al., 1992). As cited above in the case of IL-6, this finding indicates the amenability of IL-1 and IL-8 to down regulation by ribozymes (see Table I and Examples 11, 12, and 13).

Intercellular Adhesion Molecule (ICAM) -1.

Leukocyte adhesion to psoriatic keratinocytes is necessary for the inflammatory response. Intercellular Adhesion Molecule (ICAM)-1 expression has been shown to increase in many skin conditions including psoriasis. ICAM-1 is believed to play an important role in the recruitment of circulating leukocytes to the skin. Interestingly, PUVA, which has been shown to be partially effective in the treatment of psoriasis, reduces the level of ICAM-1 mRNA in keratinocytes (Krutmann, J. et al., 1991). A specific reduction of ICAM-1 levels could be achieved by design of specific ribozymes (Table I and Example 14) directed against ICAM-1 mRNA.

Ribozyme therapeutics should prove highly useful in muting the undesirable effects of all of the above mentioned factors and many, if not all, of the complications associated with the pharmacologically pluripotent drugs currently used for psoriatic disease management can also be avoided.

The present invention will be further illustrated by reference to the following non-limiting examples:

Example 7: Ribozyme cleavage of Ran/TC4 RNA, a therapeutic target in the treatment of Psoriasis.

A 944 base pair fragment of human Ran/TC4 cDNA, including the entire open reading frame (Drivas, G.T. et al., 1990, GenBank accession # M31469), was cloned into vector pGEM3Z. The plasmid was linearized with restriction endonuclease PstI and ^{32}P (alpha-^{32}P ATP (NEN))-labelled RNA transcripts were generated using T7 RNA polymerase essentially as described by Milligan et al., (1987). The transcripts were separated and purified on a 4% 7M urea/polyacrylamide gel, localized by autoradiography and eluted from gel slices using a standard procedure (Sambrook, J. et al., 1989).

Ran/TC4-specific "hammerhead" ribozymes (sequence compositions: TC4RZ1, 5'-CCA-ACC-AAC-UGA-UGA-GUC-CGU-GAG-GAC-GAA-ACA-AGU-U-3', TC4RZ2, 5'-UUU-UCC-AGC-UGA-UGA-GUC-CGU-GAG-GAC-GAA-ACC-ACC-A-3', TC4RZ3, 5'-AUA-CAU-UGC-UGA-UGA-GUC-CGU-GAG-GAC-GAA-ACU-UAA-U-3', see Figure 4) were also prepared according to Milligan et al., (1987) using synthetic deoxyoligonucleotide templates (TC4RZ1: 5' AAC-TTG-TTT-CGT-CCT-CAC-GGA-CTC-ATC-AGT-TGG-TTG-GCT-ATA-GTG-AGT-CGT-ATT-A 3'; TC4RZ2: 5' TGG-TGG-TTT-CGT-CCT-CAC-GGA-CTC-ATC-AGC-TGG-AAA-ACT-ATA-GTG-AGT-CGT-ATT-A 3'; TC4RZ3: 5' ATT-AAG-TTT-CGT-CCT-CAC-GGA-CTC-ATC-AGC-AAT-GTA-TCT-ATA-GTG-AGT-CGT-ATT-A 3') and a short synthetic DNA oligonucleotide-"18mer"- that hybridizes to the template and generates a T7 RNA polymerase promoter (5' TAA-TAC-GAC-TCA-CTA-TAG 3'), according to the methods of Example 1. The transcribed ribozymes were separated and purified on a 15% 7M urea/polyacrylamide gel and collected by elution and ethanol precipitation.

For cleavage reactions, each ribozyme and the Ran/TC4 RNA substrate were mixed in a 1:2 molar ratio in a final volume of 10ul containing 50mM Tris-HCl (pH 7.6; either in the presence or absence of 30mM MgCl$_2$. The reactions were incubated at 37°C for 90 minutes, terminated by the addition of an equal volume of loading dye (0.25% bromophenol blue, 0.25% xylene cyanol, 50% formamide, and 30mM EDTA), heated to 90°C for 2 minutes, resolved on a 4% 7M urea/polyacrylamide gel, and autoradiographed.

As shown in Figure 5, in the presence of Mg^{++}, all three "hammerhead" ribozymes cleaved the Ran/TC4 RNA substrate, giving rise to cleavage products of expected sizes (TC4RZ1 cleaved the RNA at position 83, generating two product fragments, 865nts and 83nts in length. TC4RZ2 cleaved the RNA at position 102, producing two fragments, 846nts and 102nts in length. TC4RZ3 cleaved the RNA at position 223, resulting in two fragments, 725nts and 223nts in length.) "S" indicates uncleaved substrate, and "CP" indicates cleavage products.

Based on the demonstrated activity of the above ribozyme compositions (TC4RZ1, TC4RZ2, and TC4RZ3), they may be used to cleave Ran/TC4 mRNA both *in vitro* and *in vivo*. A therapeutically effective amount of these ribozyme compositions (10ng to 1mg) can be incorporated into a formulation consisting of a delivery vehicle or carrier such as cationic liposomes. These formulations may be applied directly to an affected area of the skin. The ribozyme composition contained in this formulation may be used to cleave the Ran/TC4 mRNA present in skin cells and thus it may modify the expression of the target protein and may thereby mitigate the cutaneous condition.

Example 8: **Ribozyme cleavage of a short sequence corresponding to a portion of the TGF-alpha mRNA, a therapeutic target in the treatment of psoriasis.**

A fragment of human TGF-alpha antisense DNA (nucleotides 174-254, Jakowlew, S.B. et al., 1988; GenBank accession #M31172) attached to the T7 RNA polymerase promoter sequence can be synthesized and ^{32}P labeled RNA transcripts (81 nucleotides in length), corresponding to a portion of human TGF-alpha mRNA may be generated. Labeled ribozyme TGFRZ1 (sequence composition 5'-GCA-CCA-AAC-UGA-UGA-GUC-CGU-GAG-GAC-GAA-ACU-GCA-G-3', see Table I), designed to cleave after nucleotide 219, can also be prepared using a synthetic deoxyoligonucleotide template (5' CTG-CAG-TTT-CGT-CCT-CAC-GGA-CTC-ATC-AGT-TTG-GTG-CC-TAT-AGT-GAG-TCG-TAT-TA 3') and a short synthetic deoxyoligonucleotide-"18mer" (5' TAA-TAC-

GAC-TCA-CTA-TAG 3'), using the methods of Example 1. For cleavage reactions, the TGFRZ1 ribozyme and the TGF-alpha RNA substrate can be mixed in a 1:2 molar ratio, and can be incubated at 37°C for 90 minutes. When ribozyme and target substrate are incubated in the presence of 30mM Mg^{++}, two cleavage products of the expected sizes of 35nts and 46nts should be produced.

The TGFRZ1 ribozyme composition may be used to cleave TGF-alpha mRNA both *in vitro* and *in vivo*. A therapeutically effective amount of this ribozyme composition (10ng to 1mg) can be incorporated into a formulation consisting of a delivery vehicle or carrier such as cationic liposomes. This formulation may be applied directly to an affected area of the skin. The ribozyme composition contained in this formulation may be used to cleave the TGF-alpha mRNA present in skin cells and thus it may modify the expression of the target protein and may thereby mitigate the cutaneous condition.

Example 9: Ribozyme cleavage of a short sequence corresponding to a portion of the TNF-alpha mRNA, a therapeutic target in the treatment of psoriasis.

A fragment of human TNF-alpha antisense DNA (nucleotides 1584-1664, Marmenout, A. et al., 1985; GenBank accession #M26331) attached to the T7 RNA polymerase promoter sequence can be synthesized and ^{32}P labeled RNA transcripts (81 nucleotides in length), corresponding to a portion of human TNF-alpha mRNA may be generated. Labeled ribozyme TNFRZ1 (sequence composition 5'-ACA-UGG-GCC-UGA-UGA-GUC-CGU-GAG-GAC-GAA-AAG-GCU-U-3', see Table I), designed to cleave after nucleotide 1629, can also be prepared using a synthetic deoxyoligonucleotide template (5' AAG-CCT-TTT-CGT-CCT-CAC-GGA-CTC-ATC-AGG-CCC-ATG-TC-TAT-AGT-GAG-TCG-TAT-TA 3') and a short synthetic deoxyoligonucleotide-"18mer" (5' TAA-TAC-GAC-TCA-CTA-TAG 3'), according to the methods of Example 1. For cleavage reactions, the TNFRZ1 ribozyme and the TNF-alpha RNA substrate can be mixed in a 1:2 molar ratio, and incubated at 37°C for 90 minutes. When ribozyme and target substrate are

incubated in the presence of 30mM Mg^{++}, two cleavage products of the expected sizes of 35nts and 46nts should be produced.

The TNFRZ1 ribozyme composition may be used to cleave TNF-alpha mRNA both *in vitro* and *in vivo*. A therapeutically effective amount of this ribozyme composition (10ng to 1mg) can be incorporated into a formulation consisting of a delivery vehicle or carrier such as cationic liposomes. This formulation may be applied directly to an affected area of the skin. The ribozyme composition contained in this formulation may be used to cleave the TNF-alpha mRNA present in skin cells and thus it may modify the expression of the target protein and may thereby mitigate the cutaneous condition.

Example 10: Ribozyme cleavage of a short sequence corresponding to a portion of the interleukin-6 mRNA, a therapeutic target in the treatment of psoriasis.

A fragment of human interleukin-6 antisense DNA (nucleotides 465-545, May, L.T. et al., 1986; GenBank accession # M14582) attached to the T7 RNA polymerase promoter sequence was synthesized and ^{32}P labeled RNA transcripts (81 nucleotides in length), corresponding to a portion of human interleukin-6 mRNA were generated. Labeled ribozyme IL6RZ1 (sequence composition 5'-GAA-CUG-GAU-CAG-CUG-AUG-AGU-CCG-UGA-GGA-CGA-AAC-UUU-GUA-C-3', see Figure 6), designed to cleave after nucleotide 510, was also prepared using a synthetic deoxyoligonucleotide template (5' GTA-CAA-AAG-TTT-CGT-CCT-CAC-GGA-CTC-ATC-AGC-TGA-TCC-AGT-TCC-TAT-AGT-GAG-TCG-TAT-TA 3') and a short synthetic deoxyoligonucleotide-"18mer" (5' TAA-TAC-GAC-TCA-CTA-TAG 3'), using the methods of Example 1. For cleavage reactions, the IL6RZ1 ribozyme and the IL6 RNA substrate were mixed in a 1:2 molar ratio in the presence or absence of 30mM $MgCl_2$. The reactions were incubated at 37°C for 90 minutes. As shown in Figure 7, when the ribozyme and target substrate were incubated in the presence of 30mM Mg^{++}, two cleavage products of the expected sizes of 35nts and 46nts were produced.

Based on the demonstrated activity of the above IL6RZ1 ribozyme composition, it may be used to cleave IL-6 mRNA both *in vitro* and *in vivo*. A therapeutically effective amount of this ribozyme composition (10ng to 1mg) can be incorporated into a formulation consisting of a delivery vehicle or carrier such as cationic liposomes. This formulation may be applied directly to an affected area of the skin. The ribozyme composition contained in this formulation may be used to cleave the IL-6 mRNA present in skin cells and thus it may modify the expression of the target protein and may thereby mitigate the cutaneous condition.

Example 11: Ribozyme cleavage of a short sequence corresponding to a portion of the IL-1-alpha mRNA, a therapeutic target in the treatment of psoriasis.

A fragment of human IL-1- alpha antisense DNA (nucleotides 182-262, Nishida, T. et al., 1987; GenBank accession #M15329) attached to the T7 RNA polymerase promoter sequence can be synthesized and ^{32}P labeled RNA transcripts (81 nucleotides in length), corresponding to a portion of human IL-1-alpha mRNA may be generated. Labeled ribozyme IL1ARZ1 (sequence composition 5'-UUG-GUU-GCC-UGA-UGA-GUC-CGU-GAG-GAC-GAA-AUA-CCA-C-3', see Table I), designed to cleave after nucleotide 227, can also be prepared using a synthetic deoxyoligonucleotide template (5' GTG-GTA-TTT-CGT-CCT-CAC-GGA-CTC-ATC-AGG-CAA-CCA-AC-TAT-AGT-GAG-TCG-TAT-TA 3') and a short synthetic deoxyoligonucleotide-"18mer" (5' TAA-TAC-GAC-TCA-CTA-TAG 3'), according to the methods of Example 1. For cleavage reactions, the IL1ARZ1 ribozyme and the IL-1-alpha RNA substrate can be mixed in a 1:2 molar ratio, and incubated at 37°C for 90 minutes. When ribozyme and target substrate are incubated in the presence of 30mM Mg^{++}, two cleavage products of the expected sizes of 35nts and 46nts should be produced.

The IL1ARZ1 ribozyme composition may be used to cleave IL-1-alpha mRNA both *in vitro* and *in vivo*. A therapeutically effective amount of this ribozyme composition (10ng to 1mg) can be incorporated into a formulation consisting of a delivery vehicle or carrier such as cationic liposomes. This formulation may be

applied directly to an affected area of the skin. The ribozyme composition contained in this formulation may be used to cleave the IL-1-alpha mRNA present in skin cells and thus it may modify the expression of the target protein and may thereby mitigate the cutaneous condition.

Example 12: **Ribozyme cleavage of a short sequence corresponding to a portion of the IL-1-beta mRNA, a therapeutic target in the treatment of psoriasis.**

A fragment of human IL-1-beta antisense DNA (nucleotides 238-318, Nishida, T. et al., 1987; GenBank accession #M15330) attached to the T7 RNA polymerase promoter sequence can be synthesized and ^{32}P labeled RNA transcripts (81 nucleotides in length), corresponding to a portion of human IL-1-beta mRNA may be generated. Labeled ribozyme IL1BRZ1 (sequence composition 5'-CAA-CAA-CUC-UGA-UGA-GUC-CGU-GAG-GAC-GAA-AGC-GGC-C-3', see Table I), designed to cleave after nucleotide 283, can also be prepared using a synthetic deoxyoligonucleotide template (5' GGC-CGC-TTT-CGT-CCT-CAC-GGA-CTC-ATC-AGA-GTT-GTT-GC-TAT-AGT-GAG-TCG-TAT-TA 3') and a short synthetic deoxyoligonucleotide-"18mer" (5' TAA-TAC-GAC-TCA-CTA-TAG 3'), using the methods of Example 1. For cleavage reactions, the IL1BRZ1 ribozyme and the IL-1-beta RNA substrate can be mixed in a 1:2 molar ratio, and incubated at 37°C for 90 minutes. When ribozyme and target substrate are incubated in the presence of 30mM Mg^{++}, two cleavage products of the expected sizes of 35nts and 46nts should be produced.

The IL1BRZ1 ribozyme composition may be used to cleave IL-1-beta mRNA both *in vitro* and *in vivo*. A therapeutically effective amount of this ribozyme composition (10ng to 1mg) can be incorporated into a formulation consisting of a delivery vehicle or carrier such as cationic liposomes. This formulation may be applied directly to an affected area of the skin. The ribozyme composition contained in this formulation may be used to cleave the IL-1-beta mRNA present in skin cells and thus it may modify

the expression of the target protein and may thereby mitigate the cutaneous condition.

Example 13: Ribozyme cleavage of a short sequence corresponding to a portion of the IL-8 mRNA, a therapeutic target in the treatment of psoriasis.

A fragment of human IL-8 antisense DNA (nucleotides 1565-1645, Mukaida., N. et al., 1989; GenBank accession #M28130) attached to the T7 RNA polymerase promoter sequence can be synthesized and ^{32}P labeled RNA transcripts (81 nucleotides in length), corresponding to a portion of human IL-8 mRNA may be generated. Labeled ribozyme IL8RZ1 (sequence composition 5'-GCU-GCC-AAC-UGA-UGA-GUC-CGU-GAG-GAC-GAA-AAG-CCA-C-3', see Table I), designed to cleave after nucleotide 1610, can also be prepared using a synthetic deoxyoligonucleotide template (5' GTG-GCT-TTT-CGT-CCT-CAC-GGA-CTC-ATC-AGT-TGG-CAG-CC-TAT-AGT-GAG-TCG-TAT-TA 3') and a short synthetic deoxyoligonucleotide-"18mer" (5' TAA-TAC-GAC-TCA-CTA-TAG 3'), according to the methods of Example 1. For cleavage reactions, the IL8RZ1 ribozyme and the IL-8 RNA substrate can be mixed in a 1:2 molar ratio, and incubated at 37°C for 90 minutes. When ribozyme and target substrate are incubated in the presence of 30mM Mg^{++}, two cleavage products of the expected sizes of 35nts and 46nts should be produced.

The IL8RZ1 ribozyme composition may be used to cleave IL-8 mRNA both *in vitro* and *in vivo*. A therapeutically effective amount of this ribozyme composition (10ng to 1mg) can be incorporated into a formulation consisting of a delivery vehicle or carrier such as cationic liposomes. This formulation may be applied directly to an affected area of the skin. The ribozyme composition contained in this formulation may be used to cleave the IL-8 mRNA present in skin cells and thus it may modify the expression of the target protein and may thereby mitigate the cutaneous condition.

Example 14: Ribozyme cleavage of a short sequence corresponding to a portion of the ICAM-1 mRNA, a therapeutic target in the treatment of psoriasis.

A fragment of human ICAM-1 antisense DNA (nucleotides 173-253, Simmons, D. et al., 1988; GenBank accession #X06990) attached to the T7 RNA polymerase promoter sequence can be synthesized and ^{32}P labeled RNA transcripts (81 nucleotides in length), corresponding to a portion of human ICAM-1 mRNA may be generated. Labeled ribozyme ICMRZ1 (sequence composition 5'-GCA-GGA-GCC-UGA-UGA-GUC-CGU-GAG-GAC-GAA-AUC-CUU-U-3', see Table I), designed to cleave after nucleotide 218, can also be prepared using a synthetic deoxyoligonucleotide template (5' AAA-GGA-TTT-CGT-CCT-CAC-GGA-CTC-ATC-AGG-CTC-CTG-CC-TAT-AGT-GAG-TCG-TAT-TA 3') and a short synthetic deoxyoligonucleotide-"18mer" (5' TAA-TAC-GAC-TCA-CTA-TAG 3'), using the methods of Example 1. For cleavage reactions, the ICMRZ1 ribozyme and the ICAM-1 RNA substrate can be mixed in a 1:2 molar ratio, and incubated at 37°C for 90 minutes. When ribozyme and target substrate are incubated in the presence of 30mM Mg^{++}, two cleavage products of the expected sizes of 35nts and 46nts should be produced.

The ICMRZ1 ribozyme composition may be used to cleave ICAM-1 mRNA both *in vitro* and *in vivo*. A therapeutically effective amount of this ribozyme composition (10ng to 1mg) can be incorporated into a formulation consisting of a delivery vehicle or carrier such as cationic liposomes. This formulation may be applied directly to an affected area of the skin. The ribozyme composition contained in this formulation may be used to cleave the ICAM-1 mRNA present in skin cells and thus it may modify the expression of the target protein and may thereby mitigate the cutaneous condition.

Alopecia and Hirsutism.

The presence or absence of hair and hair color are important elements in human social communication. Two common conditions are male pattern baldness (androgenetic alopecia, AA), and in women, the opposite situation, hirsutism. The latter condition can range from the purely aesthetic, to disorders of androgen excess (hyperandrogenism) with real medical consequences (such as ovarian cysts). To date the only drug with demonstrated ability to reverse hair loss in controlled clinical trials is minoxidil.

The modest hair growth and retention reported in those who responded favorably to the drug is transient; hair loss is usually apparent approximately two months after discontinuation of treatment.

Androgens are the primary regulators of hair growth. Testosterone is converted to 5-alpha-dihydrotestosterone (5-alpha-DHT) by the enzyme 5-alpha-reductase (5-alpha-R). Both testosterone and 5-alpha-DHT can bind to androgen receptors (Mainnaring, W.I.P., 1987), but the latter is thought to play a more important growth regulatory role in hair follicles (Imperato-McGinley, J. et al., 1984). Two closely related isozymes of 5-alpha-R, 5-alpha-R1 and 5-alpha-R2, have been cloned (Andersson, S. and Russel, D.W., 1990; Andersson, S. et al., 1991).

Men with a genetically predisposed loss of 5-alpha-R, specifically 5-alpha-R2, but whose testosterone levels are normal, do not experience beard growth or pattern baldness (McPhaul, M.J. and Marcelli, M., 1992; Imperato-McGinley, J. et al., 1974). This finding along with the fact that the growth of pubic hair in these men is not affected suggests that the levels of 5-alpha-R2 and consequently the levels of 5-alpha-DHT modulate hair growth in a tissue specific manner. Evidence for the direct involvement of 5-alpha-R in male pattern baldness is that 5-alpha-R activity is greater in follicles from the balding scalp compared with non-balding areas (Sweikert, H.U. and Wilson, J.D., 1972).

Taken together, these studies point to the modulation of 5-alpha-R2 by topically applied ribozymes as an effective treatment for male pattern baldness. Ribozyme molecules designed against 5-alpha-R2 mRNA should effectively lower this enzyme's level and inhibit the onset of male pattern baldness (see Figure 8 and Example 15).

These ribozymes would also be useful in preventing hirsutism (e.g. unwanted facial hair growth in males and females). Since 5-alpha-DHT seems to increase facial hair growth, topical ribozyme inactivation of 5-alpha-R should reduce hirsutism.

Acne.

Acne vulgaris, an inflammatory condition of the pilosebaceous units of the face and trunk, is a disease whose clinical manifestations can range from minor comedones to disfiguring nodules and cysts. The pathogenesis of acne involves a complex interplay of at least four etiological factors: 1) increased sebum production by the sebaceous glands, in response to androgens; 2) ductal hypercornification, leading to obstruction; 3) increased colonization of the duct by *Propionibacterium acnes*; and 4) inflammation triggered in part, by *P. acnes* products.

Present therapeutic modalities include agents to normalize keratinization, decrease sebaceous gland activity, decrease the *P. acnes* population, and reduce inflammation. Common drugs include retinoids, antibiotics, and steroids. Of these, the retinoids have perhaps the greatest beneficial effects by influencing the four interrelated pathogenic factors of acne. The efficacy of antibiotics is being challenged as the incidence of antibiotic resistant *P. acnes*, and in particular multiply resistant strains increases. In addition, one of the primary comedolytic agents used for self-treatment, benzoyl peroxide, has been demonstrated to enhance the development of carcinomas from papillomas in mice, which raises concerns for its chronic usage.

Androgen stimulation is a prerequisite for acne (reviewed in Acne, by W.J. Cunliffe, 1989.). Androgens stimulate the sebaceous gland directly, resulting in an increase in gland size and sebum excretion rate. Sebum excretion is significantly higher in acne patients and this increase is related to the severity of the disease. The androgens testosterone and 5-alpha dihydrotestosterone (DHT) have the most potent effect on sebum production, with DHT being 2-3 times more active than testosterone. This suggests that modulation of the levels of the 5α-reductase enzyme or the DHT receptor within these target cells will result in a significant reduction of sebum production. The roles of these molecules in androgen stimulation and the ribozyme molecules that can be used to inactivate them were described in the section on alopecia and hirsutism.

The present invention will be further illustrated by reference to the following non-limiting examples:

Example 15: Ribozyme cleavage of a short sequence corresponding to a portion of the 5-alpha reductase II mRNA, a therapeutic target in the treatment of alopecia, hirsutism, and acne.

A fragment of human 5-alpha-reductase II antisense DNA (nucleotides 625-706; Andersson, S. et al., 1991, Genbank accession # M74047) attached to the T7 RNA polymerase promoter sequence was synthesized, and ^{32}P labeled RNA transcripts (82 nucleotides in length), corresponding to a portion of human 5-alpha reductase II mRNA were generated. A labeled ribozyme 5ARRZ1 (sequence composition 5'-AGU-GCU-GGG-AGG-CUG-AUG-AGU-CCG-UGA-GGA-CGA-AAC-CAA-GUG-GC-3', see Figure 8), designed to cleave after nucleotide 656, was also prepared using a synthetic deoxyoligonucleotide template (5' GCC-ACT-TGG-TTT-CGT-CCT-CAC-GGA-CTC-ATC-AGC-CTC-CCA-GCA-CTC-TAT-AGT-GAG-TCG-TAT-TA 3') and a short synthetic deoxyoligonucleotide-"18mer" (5' TAA-TAC-GAC-TCA-CTA-TAG 3'), according to the methods of Example 1. Both ribozyme and substrate were separated and purified. For cleavage reactions, the 5ARRZ1 ribozyme and 5-alpha reductase II RNA substrate were mixed in a 1:2 molar ratio in the presence or absence of 30mM $MgCl_2$. The reactions were incubated at 37°C for 90 minutes. As shown in Figure 9, when the ribozyme and target substrate were incubated in the presence of 30mM Mg^{++}, two cleavage products of the expected sizes of 32nts and 50nts were produced.

Based on the demonstrated activity of the above 5ARRZ1 ribozyme composition, it may be used to cleave 5-alpha reductase II mRNA both *in vitro* and *in vivo*. A therapeutically effective amount of this ribozyme composition (10ng to 1mg) can be incorporated into a formulation consisting of a delivery vehicle or carrier such as cationic liposomes. This formulation may be applied directly to an affected area of the skin. The ribozyme composition contained in this formulation may be used to cleave

the 5-alpha reductase II mRNA present in skin cells and thus it may modify the expression of the target protein and may thereby mitigate the cutaneous condition.

Pigmentation.

The amount of melanin in the epidermis is regulated both by its production within melanocytes and its degradation within keratinocytes. Tyrosinase is the key enzyme in the biosynthesis of melanin, although additional enzymatic activities that modify the quantity or quality of melanin have been identified, including: a dopachrome tautomerase, a peroxidase, and a gamma-glutamyl transpeptidase. Some of these activities are believed to be products of the *brown* and *slaty* loci, which encode proteins that share many features with tyrosinase (Kwon, B.S. et al., 1987a; Jimenez, M. et al., 1991; Cohen, T. et al., 1990; Hearing, V.J. and Tsukamoto, K., 1991). Characterization of the pmel 17 gene product has indicated that it may be a positive regulator of the later steps of melanogenesis (Kwon, B.S. et al., 1987b).

The use of ribozymes directed against the tyrosinase (Table I and Example 16), pmel 17 (Kwon, B.S. et al., 1991), or tyrosinase-related genes or gene products should result in a decrease in melanin production. Ribozyme molecules can be designed to effectively decrease the synthesis of these proteins by melanocytes. These reagents could be used for the treatment of hyperpigmentation conditions such as melasma, freckling, old age spots, and lentigo. Current treatment for these conditions is the application of 2-4% hydroquinone, which is generally safe, but often has a less than satisfactory effect (Engasser, P.G. and Maribach, H.I., 1981).

The fate of dopaquinone, the product of the tyrosinase reaction, is dependant upon the sulfhydryl content of melanocytes. In the presence of sulhydryl compounds, dopaquinone is converted to cysdopas (condensation products of quinone and sulhydryl compounds), and subsequently into pheomelanins--(yellow to reddish brown pigments) rather than the darker eumelanins. Two types of thiols, glutathione (GSH) and cysteine, are involved in the formation of cysdopas. Therefore, enzymes which influence the

redox state of the glutathione system (e.g. GSH reductase) can affect the level of melanization (Prota, G., 1980;1993).

Reduction of GSH levels in melanocytes could be achieved by use of ribozymes designed to inhibit the synthesis of GSH reductase (Table I and Example 17). The inhibition of GSH reductase should result in the increased production of the darker eumelanin type pigments as a result of the "shunting" of dopaquinone into this biosynthetic pathway. This should lead to a "darkening" or tanning effect of the areas of the hair or skin to which these reagents have been applied.

The present invention will be further illustrated by reference to the following non-limiting examples:

Example 16: Ribozyme cleavage of a short sequence corresponding to a portion of the tyrosinase mRNA, a target in the alteration of pigmentation.

A fragment of human tyrosinase antisense DNA (nucleotides 1177-1257, Kikuchi, H. et al., 1989; GenBank accession #X16073) attached to the T7 RNA polymerase promoter sequence can be synthesized and ^{32}P labeled RNA transcripts (81 nucleotides in length), corresponding to a portion of human tyrosinase mRNA may be generated. Labeled ribozyme TYRRZ1 (sequence composition 5'-AAG-GCC-ACC-UGA-UGA-GUC-CGU-GAG-GAC-GAA-AUC-CCG-G-3', see Table I), designed to cleave after nucleotide 1222, can also be prepared using a synthetic deoxyoligonucleotide template (5' CCG-GGA-TTT-CGT-CCT-CAC-GGA-CTC-ATC-AGG-TGG-CCT-TC-TAT-AGT-GAG-TCG-TAT-TA 3') and a short synthetic deoxyoligonucleotide-"18mer" (5' TAA-TAC-GAC-TCA-CTA-TAG 3'), according to the methods of Example 1. For cleavage reactions, the TYRRZ1 ribozyme and the tyrosinase RNA substrate can be mixed in a 1:1 molar ratio, and incubated at 37°C for 90 minutes. When ribozyme and target substrate are incubated in the presence of 10mM Mg^{++}, two cleavage products of the expected sizes of 35nts and 46nts should be produced.

The TYRRZ1 ribozyme composition may be used to cleave tyrosinase mRNA both *in vitro* and *in vivo*. A therapeutically effective amount of this ribozyme composition (10ng to 1mg) can be incorporated into a formulation consisting of a delivery

vehicle or carrier such as cationic liposomes. This formulation may be applied directly to an affected area of the skin. The ribozyme composition contained in this formulation may be used to cleave the tyrosinase mRNA present in skin cells and thus it may modify the expression of the target protein and may thereby mitigate the cutaneous condition.

Example 17: Ribozyme cleavage of a short sequence corresponding to a portion of the glutathione reductase mRNA, a target in the alteration of pigmentation.

A fragment of human glutathione reductase antisense DNA (nucleotides 245-325, Tutic, M. et al., 1990; GenBank accession #X15722) attached to the T7 RNA polymerase promoter sequence can be synthesized and ^{32}P labeled RNA transcripts (81 nucleotides in length), corresponding to a portion of human glutathione reductase mRNA may be generated. Labeled ribozyme GRRZ1 (sequence composition 5'-ACA-CAU-CCC-UGA-UGA-GUC-CGU-GAG-GAC-GAA-AAU-UCA-C-3', see Table I), designed to cleave after nucleotide 290, can also be prepared using a synthetic deoxyoligonucleotide template (5' GTG-AAT-TTT-CGT-CCT-CAC-GGA-CTC-ATC-AGG-GAT-GTG-TC-TAT-AGT-GAG-TCG-TAT-TA 3') and a short synthetic deoxyoligonucleotide-"18mer" (5' TAA-TAC-GAC-TCA-CTA-TAG 3'), according to the methods of Example 1. For cleavage reactions, the GRRZ1 ribozyme and the glutathione reductase RNA substrate can be mixed in a 1:2 molar ratio, and incubated at 37 C for 90 minutes. When ribozyme and target substrate are incubated in the presence of 30mM Mg^{++}, two cleavage products of the expected sizes of 35nts and 46nts should be produced.

The GRRZ1 ribozyme composition may be used to cleave glutathione reductase mRNA both *in vitro* and *in vivo*. A therapeutically effective amount of this ribozyme composition (10ng to 1 mg) can be incorporated into a formulation consisting of a delivery vehicle or carrier such as cationic liposomes. This formulation may be applied directly to an affected area of the skin. The ribozyme composition contained in this formulation may be used to cleave the glutathione reductase mRNA present in skin

cells and thus it may modify the expression of the target protein and may thereby mitigate the cutaneous condition.

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We Claim:

1. A ribozyme useful for the modification of cutaneous phenotypes, comprising a nucleotide sequence selected from the group consisting of 5'-CUG-UCU-UUA-AAG-CUG-AUG-AGU-CCG-UGA-GGA-CGA-AAC-AUC-ACU-UC-3', 5'-GCU-CCC-AGC-UGA-UGA-GUC-CGU-GAG-GAC-GAA-ACA-CCC-G-3', 5'-AUC-UUA-GAC-UGA-UGA-GUC-CGU-GAG-GAC-GAA-AAC-UGA-C-3', 5'-AAG-GCG-CCC-UGA-UGA-GUC-CGU-GAG-GAC-GAA-ACC-CUG-C-3',5'-UGC-UGU-UGC-UGA-UGA-GUC-CGU-GAG-GAC-GAA-AUG-GUG-U-3', 5'-GCU-GCA-CAC-UGA-UGA-GUC-CGU-GAG-GAC-GAA-AGG-AGU-U-3', 5'-CCA-ACC-AAC-UGA-UGA-GUC-CGU-GAG-GAC-GAA-ACA-ACU-U-3', 5'-UUU-UCC-AGC-UGA-UGA-GUC-CGU-GAG-GAC-GAA-ACC-ACC-A-3', 5'-AUA-CAU-UGC-UGA-UGA-GUC-CGU-GAG-GAC-GAA-ACU-UAA-U-3', 5'-GCA-CCA-AAC-UGA-UGA-GUC-CGU-GAG-GAC-GAA-ACU-GCA-G-3', 5'-ACA-UGG-GCC-UGA-UGA-GUC-CGU-GAG-GAC-GAA-AAG-GCU-U-3', 5'-GAA-CUG-GAU-CAG-CUG-AUG-AGU-CCG-UGA-GGA-CGA-AAC-UUU-GUA-C-3', 5'-UUG-GUU-GCC-UGA-UGA-GUC-CGU-GAG-GAC-GAA-AUA-CCA-C-3', 5'-CAA-CAA-CUC-UGA-UGA-GUC-CGU-GAG-GAC-GAA-AGC-GGC-C-3', 5'-GCU-GCC-AAC-UGA-UGA-GUC-CGU-GAG-GAC-GAA-AAG-CCA-C-3', 5'-GCA-GGA-GCC-UGA-UGA-GUC-CGU-GAG-GAC-GAA-AUC-CUU-U-3', 5'-AGU-GCU-GGG-AGG-CUG-AUG-AGU-CCG-UGA-GGA-CGA-AAC-CAA-GUG-GC-3', 5'-AAG-GCC-ACC-UGA-UGA-GUC-CGU-GAG-GAC-GAA-AUC-CCG-G-3', and 5'-ACA-CAU-CCC-UGA-UGA-GUC-CGU-GAG-GAC-GAA-AAU-UCA-C-3'.

2. A composition useful for the treatment of human skin comprising an effective amount of a ribozyme of claim 1, and an acceptable carrier.

3. A method of treating the skin of a patient associated with a cutaneous phenotype comprising applying to the skin of the patient the composition of claim 2.

4. The method of claim 3 wherein the cutaneous phenotype is associated with aging.

5. The method of claim 3 wherein the cutaneous phenotype is associated with psoriasis.

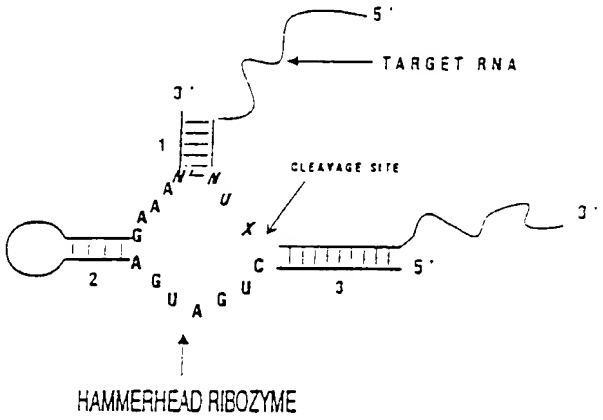
6. The method of claim 3 wherein the cutaneous phenotype is associated with alopecia.

7. The method of claim 3 wherein the cutaneous phenotype is associated with hirsutism.

8. The method of claim 3 wherein the cutaneous phenotype is associated with acne.

9. The method of claim 3 wherein the cutaneous phenotype is associated with pigmentation.

Fig. 1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N
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A
A
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2
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C
5'
3
HAMMERHEAD RIBOZYME

Fig. 2

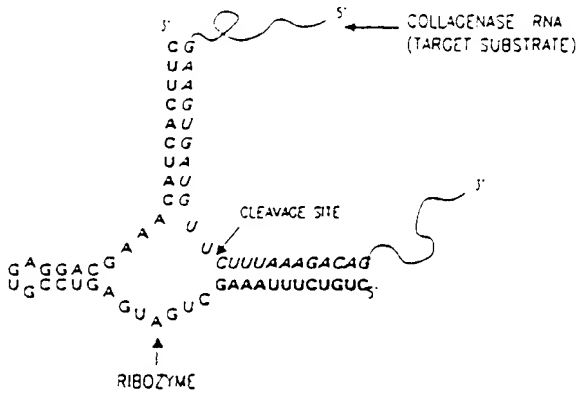


Fig. 3

Ribozyme Cleavage of Interstitial Collagenase RNA

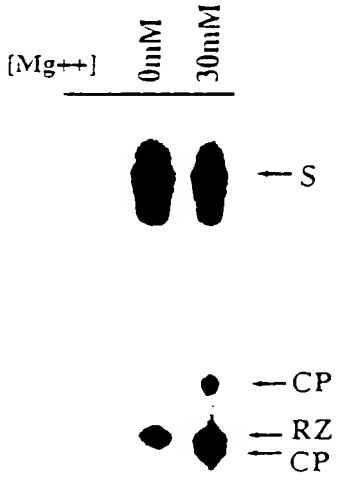
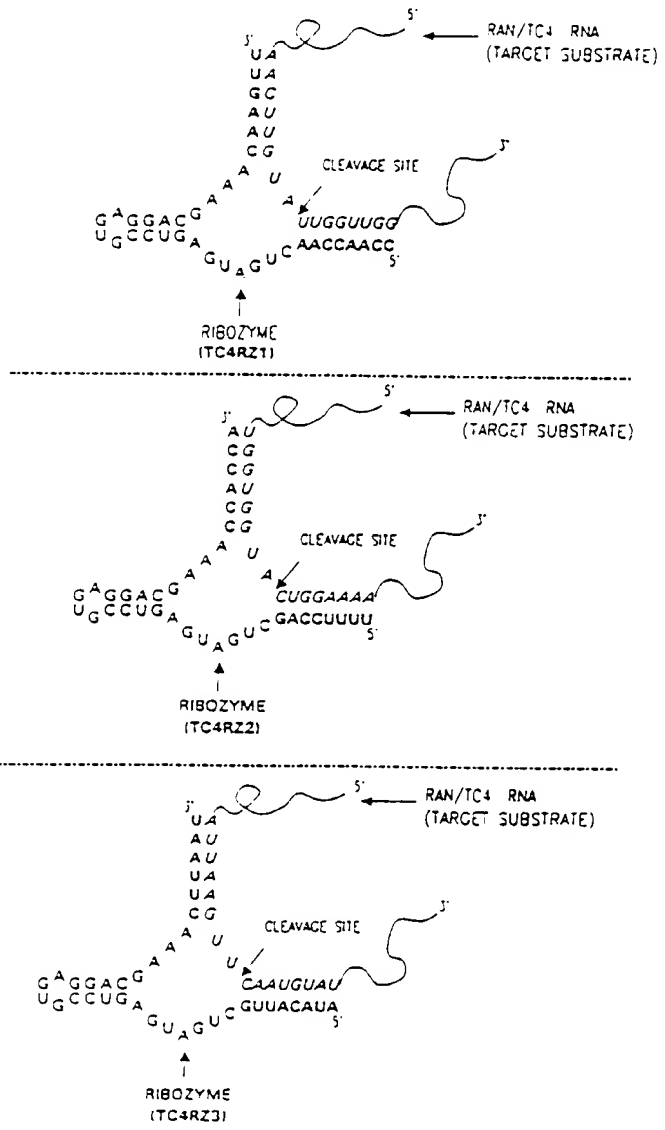


Fig. 4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RIBOZYME
(TC4RZ1)
RAN/TC4 RNA
(TARGET SUBSTRATE)
CLEAVAGE SITE
RIBOZYME
(TC4RZ2)
RAN/TC4 RNA
(TARGET SUBSTRATE)
CLEAVAGE SITE
RIBOZYME
(TC4RZ3)

Fig. 5

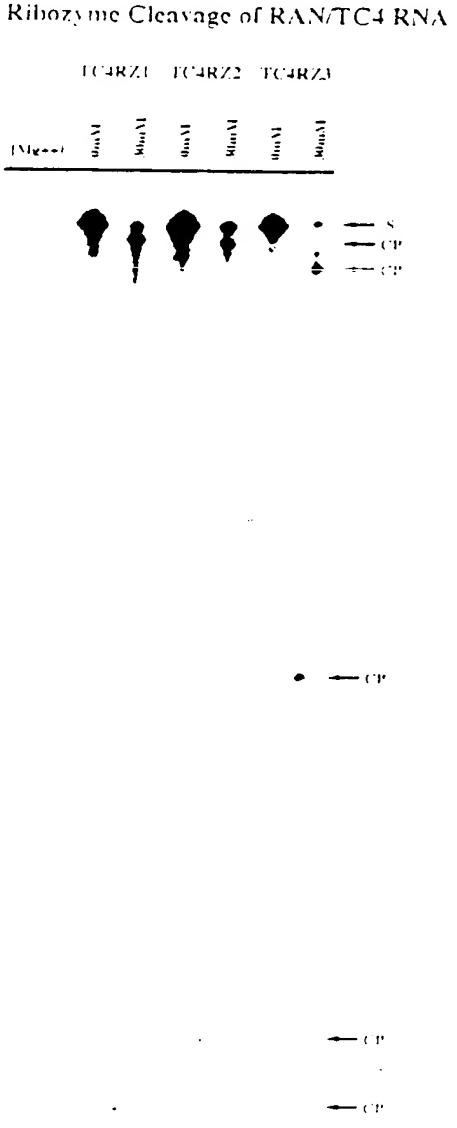

Ribozyme Cleavage of RAN/TC4 RNA
TC4RZ1
TC4RZ2
TC4RZ3

Fig. 6

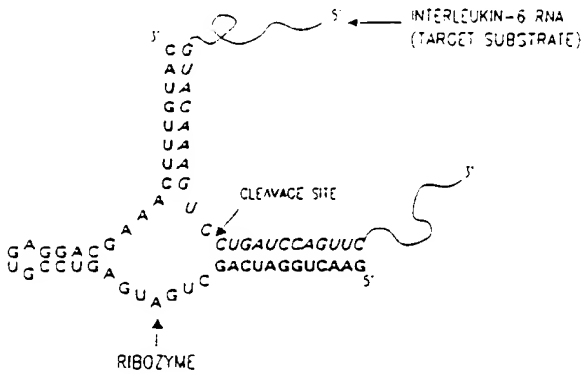


Fig. 7

Ribozyme Cleavage of Interleukin-6 RNA

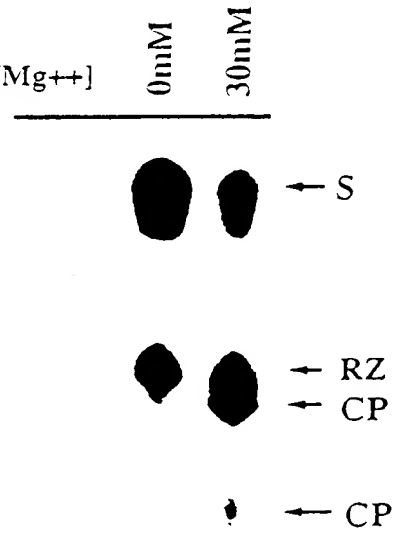


Fig. 8

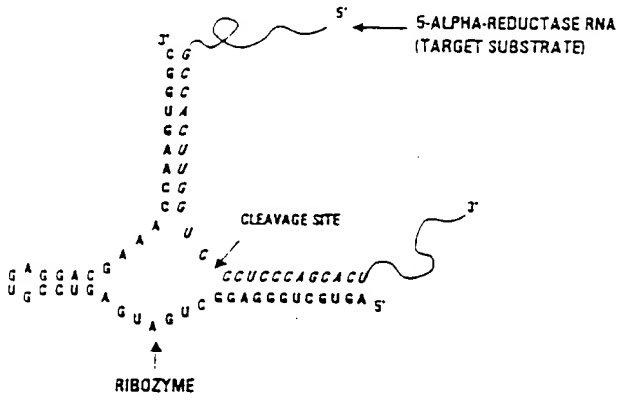
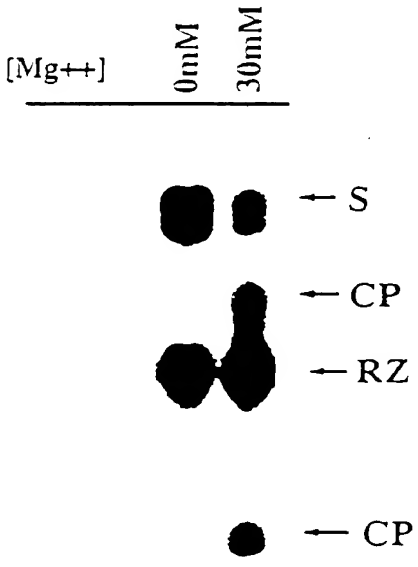


Fig. 9

Ribozyme Cleavage of
5-alpha-Reductase RNA



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/10617

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL : 435/6,91.31; 514/44; 536/23.1, 23.2, 24.5
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6,91.31; 514/44; 536/23.1, 23.2, 24.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

BIOSIS, MEDLINE, CAS, DERWENT BIOTECHNOLOGY ABSTRACTS, APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Journal of Molecular Biology, Vol. 223, issued 1992, Siuod et al., "Preformed ribozyme destroys tumor necrosis factor mRNA in human cells", pages 831-835, see entire document.	1-9
Y	Journal of Biological Chemistry, Vol. 261, No. 14, issued 15 May 1986, Goldberg et al., "Human fibroblast collagenase: Complete primary structure and homology to an oncogene transformation-induced rat protein", pages 6600-6605, see entire document.	1-9

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
31 OCTOBER 1994	11 JAN 1995

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	John LeGUYADER
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/10617

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Proc. Natl. Acad. Sci. USA, Vol. 90, issued July 1993, Yu et al., "A hairpin ribozyme inhibits expression of diverse strains of human immunodeficiency virus type 1", pages 6340-6344, see entire document.	1-9
Y	Journal of Biological Chemistry, Vol. 265, No. 28, issued 05 October 1990, Saxena et al., "Ribozymes correctly cleave a model substrate and endogenous RNA *in vitro*", pages 17106-17109, see entire document.	1-9
Y	EMBO Journal, Vol. 11, No. 12, issued 1992, Huillier et al., "Cytoplasmic delivery of ribozymes leads to efficient reduction in alpha-lactalbumin mRNA levels in C1271 mouse cells", pages 4411-4418, see entire document.	1-9
Y	EMBO Journal, Vol. 11, No. 4, issued 1992, Steinecke et al., "Expression of a chimeric ribozyme gene results in endonucleolytic cleavage of target mRNA and a concomitant reduction of gene expression *in vivo*", pages 1525-1530, see entire document.	1-9

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/10617

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

C07H 21/02; C12N 9/22, 15/00